Stabilization of Bovine Intestine Alkaline Phosphatase by Sugars

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Bovine intestine alkaline phosphatase (BIALP) is widely used as a signaling enzyme in sensitive assays such as enzyme immunoassay. In this study, we evaluated the effects of sugars on the kinetic stability of BIALP in the hydrolysis of p-nitrophenylphosphate (pNPP). The temperatures reducing initial activity by 50% in a 30-min incubation, $T_{50}$, of BIALP with 1.0 M disaccharide (sucrose and trehalose) or 2.0 M monosaccharide (glucose and fructose) were 55.0–55.5°C. Thermodynamic analysis revealed that the stabilization of BIALP by sugars was driven by the increase in the enthalpy change of activation for thermal inactivation of BIALP. No sugars affected the $k_{cat}$ of BIALP in the hydrolysis of pNPP. These results suggest that not only trehalose, which is considered the most effective stabilizer of enzymes, but also sucrose, glucose, and fructose can be used as stabilizers of BIALP.

Key words: bovine intestine alkaline phosphatase; enzyme; stability; sugar; trehalose

Alkaline phosphatase (ALP) (EC 3.1.3.1) from bovine intestine, BIALP, is a homodimeric metalloenzyme that catalyzes the hydrolysis of phosphomonoesters.1) The subunit has a molecular mass of about 50 kDa and contains two Zn$^{2+}$ ions and one Mg$^{2+}$ ion. BIALP has the highest specific activity among mammalian ALPs and is applied as a signaling enzyme in sensitive assays such as enzyme immunoassay (EIA), Western blotting analysis, nucleic acid hybridization assay, polymerase chain reaction, etc.2–5) Like other mammalian ALPs, it is expressed as a glycosylphosphatidylinositol (GPI)-containing form and is then cleaved by several phospholipases into a soluble form. Soluble and GPI-anchored BIALPs have the same activity, but the former is less stable: the temperature reducing initial activity by 50% in a 5-min incubation, $T_{50}$, of soluble BIALP was 60°C, while that of GPI-anchored BIALP was 70°C.6) In sensitive assays with BIALP as signaling enzyme, increases in performance and a prolonged shelf life are highly desired. Hence activation and stabilization of BIALP remain important goals.

In general, two schemes, Schemes 1 and 2, are used to evaluate the stabilities of enzymes:

Scheme 1

$N \rightarrow D$

Scheme 2

$N \rightarrow PD \rightarrow D$

where $N$, $D$, and $PD$ represent native, denatured, and partially denatured species respectively. Scheme 1 is applied to thermodynamic stability, in which the stability of proteins is assessed by $\Delta G_D$, which represents the difference in $G$ between the native state and the denatured state. Scheme 2 is applied to kinetic stability, in which the stability of proteins is assessed by $\Delta G^*$, which represents the difference in $G$ between the native state and the transition state.

The stabilization of enzymes by sugars and polyols has been studied based on both Schemes 1–9) and 2.10–13) Among sugars, trehalose, a natural non-reducing disaccharide in which two glucose units are linked in an $\alpha1 \rightarrow \alpha1$ linkage, has been found to be the most effective.9–20) It is now widely used to stabilize not only enzymes but also various biomaterials. We have found that amines,21) aminoalcohols,21) and polyethylene glycol22) activate BIALP, suggesting that they can be used as activators of BIALP in EIA. In this study, we examined the effects of three disaccharides (sucrose, trehalose, and maltose) and two monosaccharides (glucose and fructose) on BIALP activity and stability. Because BIALP does not unfold reversibly, we analyzed its kinetic stability based on Scheme 2.

Materials and Methods

Materials. BIALP (lot no. 13609227) was purchased from Roche Diagnostics (Basel, Switzerland). The preparation was used without further purification. p-Nitrophenyl phosphate (pNPP) (lot no. M97188) was from Nacalai Tesque (Kyoto, Japan). The concentration of pNPP was determined spectrophotometrically using a molar absorption coefficient, $\epsilon_{310}$, of 10,380 M$^{-1}$ cm$^{-1}$.9) Sucrose, trehalose, maltose, glucose, and fructose were from Nacalai Tesque. All other chemicals were from Nacalai Tesque and Wako Pure Chemical (Osaka, Japan).

Hydrolysis of pNPP. BIALP-catalyzed hydrolysis of pNPP was performed as described previously.21,22) Briefly, the reaction was initiated by mixing 2.000 mL of 0.3–6.0 mM pNPP in 1.0 M diethanolamine-HCl, 1.0 mM MgCl$_2$, and 20 mM ZnCl$_2$ at pH 9.8 with 1.000 mL of 120 mM BIALP thermally treated in the presence of sugars (90 mM sucrose, 90 mM trehalose, 90 mM maltose, 180 mM glucose, or 180 mM fructose) or 120 or 240 mM BIALP thermally treated in the absence of

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Abbreviations: ALP, alkaline phosphatase; BIALP, bovine intestine alkaline phosphatase; EIA, enzyme immunoassay; pNPP, p-nitrophenyl phosphate
sugars. The initial enzyme and substrate concentrations were 40 pm and 0.2–4.0 mm respectively. The reaction was carried out at 20 °C and was measured by following the increase in absorbance at 405 nm, $A_{405}$, for 3 min with a V-550 spectrophotometer (Jasco, Tokyo).

**Irreversible thermal inactivation of BIALP.** BIALP (100 μL of 1.32 mM in a 1.5-mL tube) in 1.0 mM diethanolamine-HCl, 1.0 mM MgCl₂, and 20 μM ZnCl₂ at pH 9.8 was incubated with 1.0 mM sucrose, or 0.5, 1.0, 1.2, or 1.5 mM trehalose, or 1.0 mM maltose, or 0.5, 1.0, 2.0, or 3.0 mM glucose, or 2.0 mM fructose at a specified temperature for 0–150 min using a dry thermo-unit DTU-1B (Taisei, Saitama, Japan). To prevent vaporization of the solution in the 1.5-mL tube and condensation at the top, the top of the tube was heated at 60 °C during incubation. Then 1 mL of 1.0 mM diethanolamine-HCl, 1.0 mM MgCl₂, and 20 μM ZnCl₂ at pH 9.8 was added to the solution. This was incubated at 20 °C for 3 min. The remaining activity of BIALP in pNP hydrolysis was determined at 20 °C with initial enzyme and substrate concentrations of 40 pm and 3 mM respectively.

**Thermodynamic analysis of the irreversible thermal inactivation of BIALP.** Under the assumption that the thermal inactivation reaction of BIALP is irreversible and consists of only one step, the first-order rate constant, $k_{obs}$, of the thermal inactivation was evaluated by plotting logarithmic values of residual activity against the time of heat treatment according to eq. 1:

$$\ln B = A - k_{obs}t$$  (1)

where $A$ is the constant term (= 4.6), and $B$ is relative activity (%) defined as the ratio of the initial reaction rate at a duration for thermal incubation (= t) to that without incubation. The activation energy, $E_a$, for thermal inactivation was determined from an Arrhenius plot according to eq. 2, and the Gibbs free energy change of activation, $\Delta G^\circ$, for thermal inactivation was determined according to eq. 3:

$$\ln(k_{obs}) = A - (E_a/R)(1/T)$$  (2)

$$\Delta G^\circ = -RT\ln(k_{obs}) - \ln(RT/\mathcal{N}h)$$  (3)

where $A$, $R$, $T$, $N$, and $h$ are constant terms, the gas constant (= 8.314 K mol⁻¹), the absolute temperature in Kelvin, the Avogadro number (= 6.022 x 10²³ mol⁻¹), and the Plank constant (= 6.626 x 10⁻²⁴ J s) respectively. The enthalpy change of activation, $\Delta H^\circ$, for thermal inactivation was calculated according to eq. 4. Using estimated $\Delta G^\circ$ and $\Delta H^\circ$ values at a certain temperature, the entropy change of activation, $\Delta S^\circ$, for thermal inactivation was calculated according to eq. 5:

$$\Delta H^\circ = E_a - RT$$  (4)

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$$  (5)

**Results**

**Irreversible thermal inactivation of BIALP in the presence of 1.0 mM disaccharides and of 2.0 mM monosaccharides.**

Figure 1 shows the effects of three disaccharides (sucrose, trehalose, and maltose) and two monosaccharides (glucose and fructose) on BIALP stability at various temperatures. To make the concentration of sugars expressed as that of monosaccharide 2.0 mM, the concentration of disaccharides was set at 1.0 mM and that of monosaccharides 2.0 mM. The relative activity of BIALP was defined as the ratio of the initial reaction rate ($v_0$) under 30 min of incubation at the temperature (30–60 °C) that to 20 °C. All the plots showed sigmoid curves. The relative activities with sugar were almost 100% for incubation temperatures of 20 to 45 °C, and decreased rapidly with increases in temperature from 45 to 60 °C. The relative activities of BIALP with sugar were higher than those without sugar for all the temperatures examined. Figure 2A shows the time course of the thermal inactivation of 1.32 and 2.64 mM BIALP treated at 54 °C without sugar. The same linear relationships between the natural logarithm of the remaining activity and incubation duration were obtained, suggesting that the thermal inactivation can be regarded as a first-order reaction. Figure 2B–G shows the time course of the thermal inactivation of 1.32 mM BIALP with sugar at 52–60 °C and without sugar at 45–60 °C. The inactivation rates increased with increases in temperature. Figure 2H shows an Arrhenius plot of the first-order rate constant, $k_{obs}$, of the thermal inactivation of BIALP. A linear relationship was obtained between the natural logarithm of $k_{obs}$ and 1/T. The temperatures required to reduce initial activity by 50% in a 30-min incubation, $T_{50}$, were estimated from the Arrhenius plot, as the temperature giving the $k_{obs}$ value of 2.2 x 10⁻³ s⁻¹ according to eq. 2. As shown in Table 1, the $T_{50}$ value of BIALP without sugar was 50.3 ± 0.1 °C, and those with 1.0 mM sucrose, 1.0 mM trehalose, 2.0 mM glucose, or 2.0 mM fructose were 55.0–55.5 °C, 4.7–5.2 °C higher than without sugar. The $T_{50}$ of BIALP with 1.0 mM maltose was 53.5 ± 0.1 °C. These results indicate that all the sugars stabilize BIALP, but that the stabilizing effect of maltose is lower than those of the other four sugars.

Table 2 shows the thermodynamic parameters for the irreversible thermal inactivation of BIALP. The activation energy, $E_a$, of BIALP with 1.0 mM sucrose, 1.0 mM trehalose, 2.0 mM glucose, or 2.0 mM fructose was 282–308 kJ mol⁻¹, 69–95 kJ mol⁻¹ higher than without sugar (213 ± 6 kJ mol⁻¹). The enthalpy change of activation, $\Delta H^\circ$, of BIALP with these four sugars was 280–306 kJ mol⁻¹, 70–96 kJ mol⁻¹ higher than without sugar (210 ± 6 kJ mol⁻¹), and the entropy change of activation, $\Delta S^\circ$, of BIALP with these four sugars was 542–621 J mol⁻¹ K⁻¹, 208–287 J mol⁻¹ K⁻¹ higher than without sugar (334 ± 12 J mol⁻¹ K⁻¹). Maltose also increased the $E_a$, $\Delta H^\circ$, and $\Delta S^\circ$ of BIALP, but the extents of increase were less than those for the other four sugars. These results indicate that all sugars stabilize BIALP by increasing $\Delta H^\circ$ more than $T\Delta S^\circ$. 
Irreversible thermal inactivation of BIALP in the presence of 0.5–1.5 m trehalose and of 0.5–3.0 m glucose

We examined the effects of the trehalose (0.5–1.5 m) and glucose (0.5–3.0 m) concentrations on their stabilizing effects. The results for 1.5 m trehalose and 3.0 m glucose are shown in Fig. 3A and B respectively, but those for 0.5 and 1.2 m trehalose and 0.5 and 1.0 m glucose are not shown. All the results are summarized in Table 1. A linear relationship was again obtained between the natural logarithm of the remaining activity and the incubation duration. BIALP incubated at 58°C for 30 min with 1.5 m trehalose retained about 60% of its original activity (hollow square in Fig. 3A), while that incubated at 58°C for 15 min with 3.0 m glucose retained 30% of its original activity (hollow square in Fig. 3B), indicating that 1.5 m trehalose has a stronger stabilizing effect than 3.0 m glucose. Figure 3C shows an Arrhenius plot of the \( k_{obs} \) of BIALP with 1.5 m trehalose and with 3.0 m glucose. A linear relationship was obtained between the natural logarithm of \( k_{obs} \) and \( 1/T \).
the Arrhenius plot, the $T_{50}$ of BIALP with 1.5 M trehalose was estimated to be $58.4 \pm 0.3$°C, 3.4°C higher than with 1.0 M trehalose (55.0 ± 0.1°C), while the $T_{50}$ of BIALP with 3.0 M glucose was estimated to be $54.1 \pm 0.2$°C, almost the same as with 2.0 M glucose (55.5 ± 0.1°C). These results indicate that the stabilizing effect of trehalose increases with increasing concentrations from 1.0 to 1.5 M, while that of glucose does not increase with increasing concentrations from 2.0 to 3.0 M.

**Irreversible thermal inactivation of BIALP in the presence of 0–1.5 M disaccharides**

The stabilizing effect of 1.0 M maltose was less than those of 1.0 M sucrose, 1.0 M trehalose, 2.0 M glucose, and 2.0 M sucrose (Figs. 1 and 2 and Table 1). We examined the stabilizing effect of maltose at various concentrations in a range of 0–1.5 M (Fig. 4). The relative activities of BIALP increased with increasing disaccharide concentrations, and those with sucrose or trehalose were higher than those with maltose at all the concentrations examined. The concentrations required to reduce initial activity by 50% were estimated to be 0.7 M for sucrose and trehalose and 1.3 M for maltose. This indicates that the stabilizing effect of maltose is lower than those of sucrose and trehalose.

### Table 1. $T_{50}$ of BIALP

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$T_{50}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without</td>
<td>50.3 ± 0.1</td>
</tr>
<tr>
<td>1.0 M sucrose</td>
<td>55.1 ± 0.1</td>
</tr>
<tr>
<td>0.5 M trehalose</td>
<td>53.5 ± 0.1</td>
</tr>
<tr>
<td>1.0 M trehalose</td>
<td>55.0 ± 0.1</td>
</tr>
<tr>
<td>1.2 M trehalose</td>
<td>56.2 ± 0.1</td>
</tr>
<tr>
<td>1.5 M trehalose</td>
<td>58.4 ± 0.3</td>
</tr>
<tr>
<td>1.0 M maltose</td>
<td>53.5 ± 0.1</td>
</tr>
<tr>
<td>0.5 M glucose</td>
<td>50.3 ± 0.1</td>
</tr>
<tr>
<td>1.0 M glucose</td>
<td>53.6 ± 0.1</td>
</tr>
<tr>
<td>2.0 M glucose</td>
<td>55.5 ± 0.1</td>
</tr>
<tr>
<td>3.0 M glucose</td>
<td>54.1 ± 0.2</td>
</tr>
<tr>
<td>2.0 M fructose</td>
<td>55.5 ± 0.1</td>
</tr>
</tbody>
</table>

*Temperatures reducing initial activity by 50% under 30 min of incubation. $T_{50}$ was estimated from an Arrhenius plot as the temperature giving a $k_{obs}$ value of $2.2 \times 10^{-3}$ s⁻¹ according to eq. 2. Averages of triplicate determinations with SD values are shown.

### Table 2. Thermodynamic Parameters for Thermal Inactivation of BIALP

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$E_a$ (kJ mol⁻¹)</th>
<th>$\Delta G^\ddagger$ (kJ mol⁻¹)</th>
<th>$\Delta H^\ddagger$ (kJ mol⁻¹)</th>
<th>$\Delta S^\ddagger$ (J mol⁻¹ K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without</td>
<td>213 ± 6</td>
<td>98 ± 1</td>
<td>210 ± 6</td>
<td>334 ± 12</td>
</tr>
<tr>
<td>1.0 M sucrose</td>
<td>290 ± 7</td>
<td>99 ± 1</td>
<td>288 ± 5</td>
<td>567 ± 14</td>
</tr>
<tr>
<td>0.5 M trehalose</td>
<td>265 ± 7</td>
<td>99 ± 1</td>
<td>262 ± 5</td>
<td>532 ± 31</td>
</tr>
<tr>
<td>1.0 M trehalose</td>
<td>293 ± 8</td>
<td>99 ± 1</td>
<td>291 ± 8</td>
<td>575 ± 15</td>
</tr>
<tr>
<td>1.2 M trehalose</td>
<td>288 ± 17</td>
<td>102 ± 1</td>
<td>285 ± 15</td>
<td>550 ± 30</td>
</tr>
<tr>
<td>1.5 M trehalose</td>
<td>275 ± 25</td>
<td>102 ± 1</td>
<td>269 ± 24</td>
<td>502 ± 49</td>
</tr>
<tr>
<td>1.0 M maltose</td>
<td>246 ± 4</td>
<td>98 ± 1</td>
<td>243 ± 3</td>
<td>436 ± 10</td>
</tr>
<tr>
<td>0.5 M glucose</td>
<td>214 ± 1</td>
<td>99 ± 1</td>
<td>211 ± 7</td>
<td>340 ± 15</td>
</tr>
<tr>
<td>1.0 M glucose</td>
<td>295 ± 1</td>
<td>99 ± 1</td>
<td>292 ± 10</td>
<td>538 ± 41</td>
</tr>
<tr>
<td>2.0 M glucose</td>
<td>308 ± 4</td>
<td>99 ± 1</td>
<td>306 ± 3</td>
<td>621 ± 10</td>
</tr>
<tr>
<td>3.0 M glucose</td>
<td>269 ± 17</td>
<td>100 ± 1</td>
<td>267 ± 16</td>
<td>502 ± 40</td>
</tr>
<tr>
<td>2.0 M fructose</td>
<td>282 ± 4</td>
<td>99 ± 1</td>
<td>280 ± 3</td>
<td>542 ± 12</td>
</tr>
</tbody>
</table>

$E_a$ is the activation energy. $\Delta G^\ddagger$, $\Delta H^\ddagger$, and $\Delta S^\ddagger$ are the Gibbs free energy change of activation, the enthalpy change of activation, and the entropy change of activation respectively at 60°C. Averages of triplicate determinations with SD values are shown.

The relative activity of BIALP was defined as the ratio of $v_{rel}$ to that without incubation. A and B, Time courses of thermal inactivation. BIALP at 1.32 nm was incubated at 52–64°C in the presence of 1.5 M trehalose (A) or 3.0 M glucose (B) for 0–75 min. The $k_{obs}$ value was estimated from the slope. Symbols: 52°C, solid circle; 54°C, hollow circle; 56°C, solid square; 58°C, hollow square; 60°C, solid inverted triangle; 62°C, hollow inverted triangle; and 64°C, solid diamond. The $k_{obs}$ values in A at 58, 60, 62, and 64°C were 0.333 × 10⁻³, 0.653 × 10⁻³, 1.14 × 10⁻³, and 1.95 × 10⁻³ s⁻¹ respectively. In B at 52, 54, 56, and 58°C were 0.203 × 10⁻³, 0.387 × 10⁻³, 0.665 × 10⁻³, and 1.26 × 10⁻³ s⁻¹, respectively. The broken line indicates the relative activity of 50%.

C, Arrhenius plot of $k_{obs}$ values. Symbols: 1.5 M trehalose, hollow circle; 3.0 M glucose, solid circle.

### Fig. 3. Irreversible Thermal Inactivation of BIALP in the Presence of 1.5 M Trehalose and of 3.0 M Glucose.

The relative activity of BIALP was defined as the ratio of $v_{rel}$ to that without incubation. A and B, Time courses of thermal inactivation. BIALP at 1.32 nm was incubated at 52–64°C in the presence of 1.5 M trehalose (A) or 3.0 M glucose (B) for 0–75 min. The $k_{obs}$ value was estimated from the slope. Symbols: 52°C, solid circle; 54°C, hollow circle; 56°C, solid square; 58°C, hollow square; 60°C, solid inverted triangle; 62°C, hollow inverted triangle; and 64°C, solid diamond. The $k_{obs}$ values in A at 58, 60, 62, and 64°C were 0.333 × 10⁻³, 0.653 × 10⁻³, 1.14 × 10⁻³, and 1.95 × 10⁻³ s⁻¹ respectively. In B at 52, 54, 56, and 58°C were 0.203 × 10⁻³, 0.387 × 10⁻³, 0.665 × 10⁻³, and 1.26 × 10⁻³ s⁻¹, respectively. The broken line indicates the relative activity of 50%.

C, Arrhenius plot of $k_{obs}$ values. Symbols: 1.5 M trehalose, hollow circle; 3.0 M glucose, solid circle.

### Fig. 4. Irreversible Thermal Inactivation of BIALP under 30 min of Incubation at 54°C in the Presence of 0–1.5 M Disaccharide.

The relative activity of BIALP was defined as the ratio of $v_{rel}$ under 30 min of incubation at 54°C to that at 20°C. Symbols: sucrose, solid circle; trehalose, hollow circle; maltose, solid square.
metalloprotease 7 (MMP-7) \(E_c, 237 \text{kJ mol}^{-1}\), \(T_{50}, 69 \text{C} \), \(24\) bovine erythrocyte Cu,Zn-superoxide dismutase \(E_s, 268 \text{kJ mol}^{-1}\), \(T_{50}, 77 \text{C} \), \(25\) Bacillus amyloli-quefaciens \(\alpha\)-amylase (BAA) \(E_a, 249 \text{kJ mol}^{-1}\), \(T_{50}, 75 \text{C} \), \(26\) and 0.19 \(\alpha\)-amylase inhibitor from wheat kernel \(0.19 \text{AI} \) \(E_s, 87 \text{kJ mol}^{-1}\), \(T_{50}, 88 \text{C} \). \(27\) In view of high \(E_a\) and low \(\Delta G^\circ\) values for BIALP, the calculated \(\Delta S^\circ\) of BIALP is relatively large \(334 \pm 12 \text{J mol}^{-1} \text{K}^{-1}\) as compared, for example, to those of BAA \(773 \text{J mol}^{-1} \text{K}^{-1}\) \(26\) and 0.19 AI \(78.6 \text{J mol}^{-1} \text{K}^{-1}\). \(27\)

**The mechanism of stabilization of BIALP by sugars**

Sugars increased \(\Delta H^\circ\) and \(\Delta S^\circ\) of BIALP \(2\), indicating that the stabilization of BIALP by sugars is driven by increases in \(\Delta H^\circ\), and that large \(\Delta H^\circ\) is compensated for by large \(\Delta S^\circ\). This suggests that sugars make the transition state of BIALP in the thermal inactivation more robust, but simultaneously deprive it of freedom.

As the stabilization of enzymes in aqueous solution, Sampedro and Uribe provided the viscosity theory, that the ability of sugars to increase the viscosity of a solution is the most important. \(10\) Viscosity inhibits enzyme unfolding and inactivation. In the present study, we analyzed the kinetic but not the thermodynamic stability of BIALP. Further study is required to relate the mechanism of stabilization of BIALP by sugars in aqueous solution to viscosity theory.

For the stabilization of freeze-dried enzymes, Crowe *et al.* provided the glass theory, that the ability of sugars to form glasses is the most important factor. \(15\) According to this theory, a glassy matrix protects the enzyme from inactivation. The glass transition temperature, \(T_g\), the temperature above which a transition from viscous to the fluid state occurs, of anhydrous trehalose was 113.9 °C, and those of anhydrous sucrose, maltose, glucose, and fructose were 60, 80, 30, and 0 °C respectively. \(15\) This theory explains why trehalose is the most effective stabilizer of freeze-dried enzymes. Unlike the stabilization of freeze-dried enzymes, our results suggest that not only trehalose but also sucrose, glucose, and fructose stabilize BIALP, and that the effect of trehalose is the largest.

**References**